

# An Investigational Antiviral Drug, DAS181, Effectively Inhibits Replication of Zoonotic Influenza A Virus Subtype H7N9 and Protects Mice From Lethality

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**Human infections caused by avian influenza A virus type subtype H7N9 have been associated with substantial morbidity and mortality. Emergence of virus variants carrying markers of decreased susceptibility to neuraminidase inhibitors was reported. Here we show that DAS181 (Fludase), an antiviral drug with sialidase activity, potently inhibited replication of wild-type influenza A(H7N9) and its oseltamivir-resistant R292K variants in mice. A once-daily administration initiated early after lethal infection hampered body weight loss and completely protected mice from lethality. We observed a time-dependent effect for 24–72-hour delayed DAS181 treatments on morbidity and mortality. The results warrant further investigation of DAS181 for influenza treatment.**

**Keywords.** H7N9; Fludase (DAS181); oseltamivir; drug resistance; R292K; neuraminidase inhibitor.

The ongoing outbreak caused by an avian influenza A virus subtype H7N9 in China, associated with substantial morbidity and mortality, has raised public health concerns [1,2]. The majority of hospitalized patients had acute respiratory syndrome,

Received 20 November 2013; accepted 13 February 2014; electronically published 25 February 2014.

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**The Journal of Infectious Diseases** 2014;210:435–40

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DOI: 10.1093/infdis/jiu105

including severe pneumonia and dyspnea [3,4]. Because of the resistance to M2 blockers, the neuraminidase (NA) inhibitors (NAIs), particularly oseltamivir, were used to mitigate disease severity among infected patients during the outbreak of influenza A(H7N9) infection. However, as with other antiviral drugs, the therapeutic benefit of NAIs can be compromised by the emergence of resistant virus variants.

In 4 influenza A(H7N9)-infected patients, emergence of virus variants with Arg292Lys (R292K) substitution in the NA (N2 numbering) was detected during antiviral treatment [3,5–7], at least one of which was a fatal case [5]. R292K was shown to be associated with decreased susceptibility to oseltamivir and other NAIs [6,8]. These findings highlight the need for new anti-influenza virus drugs that are effective against NAI-resistant virus variants.

Here we evaluated the efficacy of an investigational antiviral drug, DAS181 (Fludase), that is undergoing clinical evaluation [9]. DAS181, a recombinant fusion protein with sialidase activity [10], removes sialic acid-containing receptor from respiratory epithelial cells, preventing attachment and replication of influenza virus. DAS181 was shown to suppress replication of influenza viruses, including highly pathogenic avian influenza A virus subtype H5N1, in cell culture and mice [10–12].

## METHODS

Details of the NA inhibition assay, focus reduction assay, and statistical analysis can be found in the [Supplementary Materials](#).

### Viruses and Biological Cloning

Two egg-grown isolates of influenza A(H7N9) viruses, A/Shanghai/1/2013 (Shanghai/1) and A/Taiwan/1/2013 (Taiwan/1), were kindly shared by the China and Taiwan centers for disease control. The 2009 pandemic influenza A virus subtype H1N1 (influenza A[H1N1]pdm09) strains A/California/12/2012 (California/12) and A/Wisconsin/53/2009 (Wisconsin/53) were submitted to the World Health Organization Collaborating Center for Surveillance, Epidemiology, and Control of Influenza at the Centers for Disease Control and Prevention (CDC) for virological surveillance.

Because these influenza A(H7N9) isolates contained a mixed viral population, plaque purification was performed in MDCK-SIAT1 cells to separate the oseltamivir-resistant virus variant carrying R292K from the wild-type virus with R292. Sanger sequencing analyses of the plaque-purified viruses confirmed that R292K was the only amino acid difference between the NAs of wild-type and R292K virus variant. The HA of the Shanghai/1

R292K variant had a mixture of 151A/S (2013 H7 numbering) and 209G/E, while that of the Taiwan/1 R292K variant showed a D340G substitution, compared with characteristics of the respective wild-type viruses.

### Animals and DAS181 Treatment

All procedures were in compliance with the protocol approved by the CDC Institutional Animal Care and Use Committee. The dose that killed 50% of inoculated mice ( $MLD_{50}$ ) was determined in 6–8-week-old female BALB/c mice (4 per virus dilution; Jackson Laboratories, Bar Harbor, ME). While anesthetized by isoflurane, mice were intranasally inoculated (50  $\mu$ L per mouse) with influenza A(H7N9), using 10-fold serial dilutions of  $10^1$ – $10^6$  50% tissue culture infective doses ( $TCID_{50}$ ). Animals that lost  $\geq 25\%$  of initial body weight because of infection were humanely euthanized.

To assess the protective efficacy of DAS181 against influenza A (H7N9), groups of 16 mice were intranasally inoculated with 5  $MLD_{50}$  under the same procedures described above. DAS181 (0.3, 0.6, or 1 mg/kg) or placebo (phosphate-buffered saline) was given intranasally (50  $\mu$ L/mouse) to anesthetized mice once daily for 6 days, starting at 4 hours after infection (early treatment) or 24, 48, or 72 hours after infection (delayed treatment). For comparison, additional groups of animals were lethally challenged with California/12 virus ( $5 \times 10^4$   $TCID_{50}$  per mouse) and treated with DAS181 as described above. Details of oseltamivir treatment in mice are available in the [Supplementary Materials](#).

DAS181 was kindly provided by Ansun BioPharma (San Diego, CA). Oseltamivir phosphate (used in the *in vivo* experiment) was purchased from Sequoia Research Product (Pangbourne, United Kingdom). Oseltamivir carboxylate (used in the *in vitro* assay) was kindly provided by F. Hoffmann-La Roche (Basel, Switzerland), zanamivir by GlaxoSmithKline (Uxbridge, United Kingdom), peramivir by BioCryst Pharmaceutical (Durham, NC), and laninamivir by Biota (Victoria, Australia).

## RESULTS

### In Vitro Drug Susceptibility

The plaque-purified R292K virus variants of Shanghai/1 and Taiwan/1 influenza A(H7N9) isolates exhibited highly reduced inhibition by oseltamivir carboxylate (>10 000-fold) and peramivir (1388–1587-fold) and reduced inhibition by zanamivir (51–55-fold) and laninamivir (22–24-fold) in the NA inhibition assay ([Supplementary Table 1](#)). These results are consistent with those in previously published reports [8, 13, 14] and may be interpreted as resistance to 1 or more NAIs. The wild-type influenza A(H1N1)pdm09 isolates included as a control showed median inhibitory concentration ( $IC_{50}$ ) values similar to those of wild-type influenza A(H7N9) isolates against all 4 NAIs tested.

Next, the ability of DAS181 to inhibit replication of the R292K virus variant and its wild-type counterpart in cell culture

was tested using the focus reduction assay. Pretreatment of MDCK and MDCK-SIAT1 monolayers with DAS181 resulted in a dose-dependent reduction in the number of foci produced by the influenza A(H7N9) isolates ([Supplementary Table 2A](#) and [2B](#)). The  $IC_{50}$  values of the influenza A(H7N9) pair were similar to each other (<2-fold difference) and to those for influenza A (H1N1)pdm09, used for comparison, and ranged from  $\leq 0.25$  to 1.0 nM. This finding demonstrated the inhibitory effect of DAS181 on the replication of the influenza A(H7N9) isolates in both cell lines commonly used for influenza virus testing.

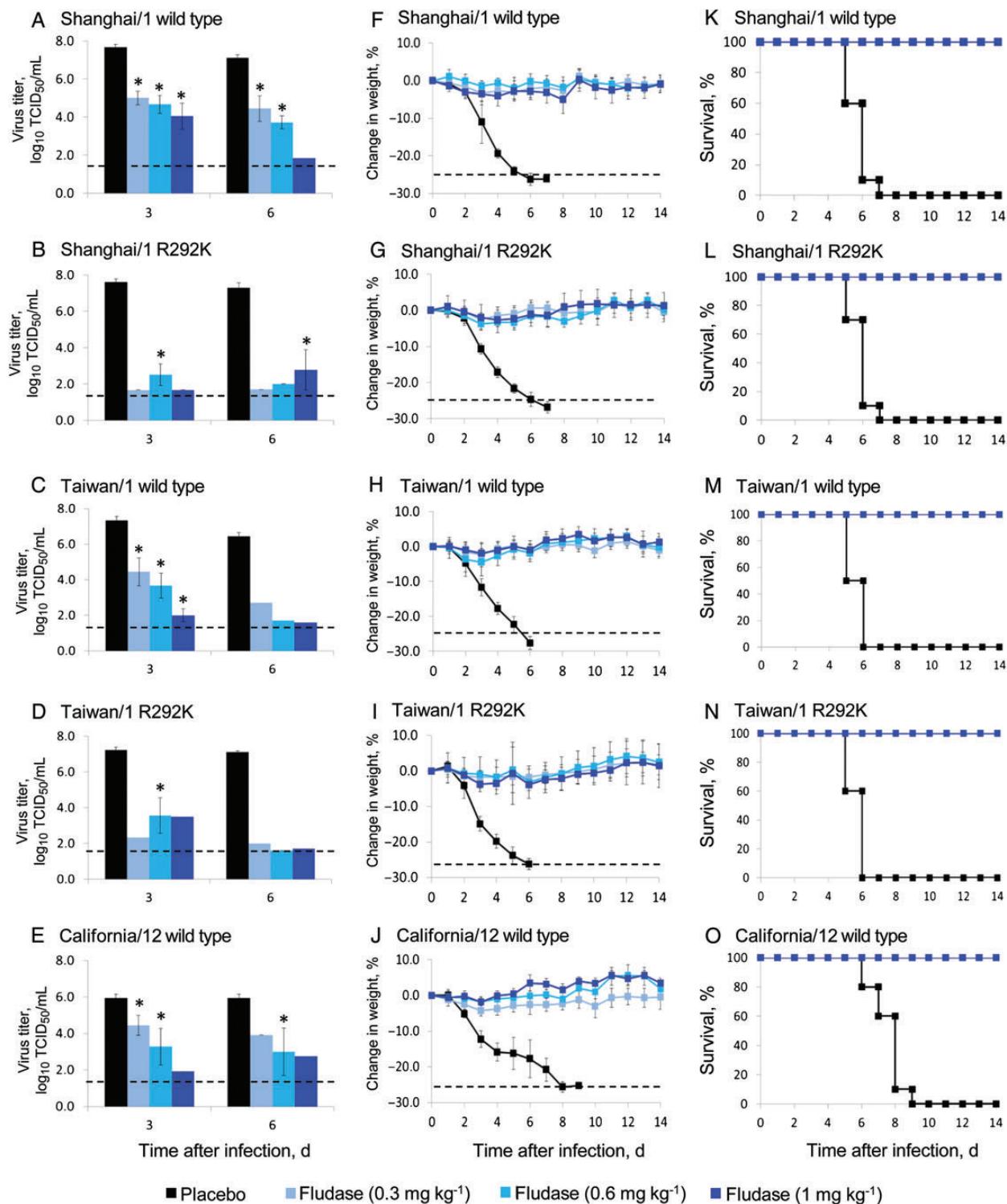
### Early Treatment of Infected Mice

To assess anti-influenza virus potency of DAS181 *in vivo*, we used a mouse lethal model. The  $MLD_{50}$  values for the Shanghai/1 wild-type and R292K virus variant were  $10^3$  and  $2.0 \times 10^3$   $TCID_{50}$ , respectively. The Taiwan/1 virus pair showed a 16–32-fold higher  $MLD_{50}$  ( $3.2 \times 10^4$   $TCID_{50}$ ), suggesting lower pathogenicity in mice.

Thereafter, animals were challenged with 5  $MLD_{50}$  of influenza A(H7N9) and treated with either placebo or DAS181, starting 4 hours after infection. Notably, in the placebo-treated group, both pairs of influenza A(H7N9) isolates replicated to comparable titers (approximately  $10^7$   $TCID_{50}/mL$ ; Figure 1A–D) in the mouse lung and caused a similar pattern (>25%) of body weight loss (Figure 1F–I). Furthermore, these animals exhibited lethargy, ruffled fur, hunched posture, and dyspnea, and all died from infection between days 6 and 9 after infection. Noteworthy, no reversion to the wild type (R292) was detected, based on pyrosequencing analysis conducted on virus in lung homogenates of animals infected with either R292K virus.

Intranasal treatment with DAS181 (0.3, 0.6, or 1 mg/kg) once daily for 6 days led to dramatic reduction ( $P \leq .02$ ) in lung virus titers of approximately  $2 \log_{10}$  on day 3 and approximately  $5 \log_{10}$  on day 6, regardless of the influenza A(H7N9) isolate used (Figure 1A–D). DAS181-treated mice showed neither remarkable disease signs nor statistically significant body weight loss ( $P > .05$ ; Figure 1F–I). Moreover, the DAS181 treatment was accompanied by the complete protection of influenza A(H7N9)-infected animals against lethality, even at the lowest drug dose used (Figure 1K–N), whereas all placebo-treated animals died from infection by day 9. Similar antiviral effects were observed in the DAS181-treated mice lethally infected with  $5 \times 10^4$   $TCID_{50}$  of influenza A(H1N1)pdm09 (Figure 1E, J, O).

Conversely, we found that oseltamivir treatment (100 mg/kg/dose) twice daily produced no or only a slight reduction (approximately 0.2–0.8  $\log_{10}$ ) in lung virus titers for all 4 influenza A(H7N9) isolates tested ([Supplementary Figure 1A–D](#)) and did not prevent mortality against the lethal challenge dose ([Supplementary Figure 1K–N](#)). In contrast, 90% of mice infected with influenza A(H1N1)pdm09 and treated with oseltamivir survived the lethal challenge, while all placebo-treated animals died from infection by day 9 ([Supplementary Figure 1J, O](#)).



**Figure 1.** Efficacy of early treatment in infected mice. Animals (16/group) were intranasally inoculated with 5 50% mouse lethal doses of plaque-purified influenza A virus subtype H7N9, equivalent to  $5 \times 10^3$  50% tissue culture infective doses (TCID<sub>50</sub>) of Shanghai/1 wild-type (A, F, K),  $10^4$  TCID<sub>50</sub> of Shanghai/1 R292K (B, G, L),  $1.6 \times 10^5$  TCID<sub>50</sub> of Taiwan/1 wild-type (C, H, M), or  $1.6 \times 10^5$  TCID<sub>50</sub> of Taiwan/1 R292K (D, I, N). California/12 virus (a 2009 pandemic influenza A virus subtype H1N1 isolate;  $5 \times 10^4$  TCID<sub>50</sub>) was used as a control (E, J, O). Mice were treated with DAS181 4 hours after infection (for a total of 6 regimens). Three mice per group were euthanized on days 3 and 6 after infection, and virus titers in lungs were determined by a TCID<sub>50</sub> assay in MDCK cells (A-E); dotted lines indicate the lower limit of virus titer detection ( $1.6 \log_{10}$  TCID<sub>50</sub>/mL). Body weights were monitored daily (F-J); animals that lost  $\geq 25\%$  (shown in dotted lines) of their initial body weights were humanely euthanized, and numbers of surviving animals are shown (K-O). *P* values of  $<.05$  (asterisks) denote statistically significant differences from values for placebo-treated groups. Error bars indicate SDs. SDs are not shown if virus was detected only in 1 or 2 of 3 lung homogenates.

### Delayed Treatment of Infected Mice

In the next experiment, mice infected with Taiwan/1 wild-type virus, its R292K variant, or influenza A(H1N1)pdm09 were treated with DAS181 starting 24, 48, or 72 hours after infection; the 2 last treatments were done at the highest DAS181 dose only. A time-dependent effect was observed on all DAS181-treated mice. The 24-hour delayed treatment warranted least morbidity and complete protection over lethal infections with either influenza A(H7N9) isolate (Figure 2A–D). Infected mice given DAS181 after 48 hours showed  $\geq 75\%$  survival, while 75% and 25% protection was seen after 72 hours in wild-type and R292K virus–infected mice, respectively. Of note, all delayed treatments completely protected mice after infection with influenza A(H1N1)pdm09 (Figure 2E and 2F).

Since we did not observe protective effect of early oseltamivir treatment against influenza A(H7N9), we reduced the challenge dose to  $10^4$  TCID<sub>50</sub> (16-fold lower than 5 MLD<sub>50</sub> or  $1.6 \times 10^5$  TCID<sub>50</sub>) of the Taiwan/1 virus pair. All placebo-treated animals survived this challenge despite approximately 20% weight loss (Figure 2H and 2I). A dose-dependent improvement of morbidity during delayed treatment with 25 or 100 mg/kg/dose oseltamivir was observed in mice infected with wild type, judged by body weight change. Furthermore, a trend toward reduction in lung virus titers of the wild-type virus was detected after oseltamivir treatment (Figure 2G). Contrariwise, no effect on body weight and lung virus titer was seen in mice infected with R292K influenza A(H7N9).

## DISCUSSION

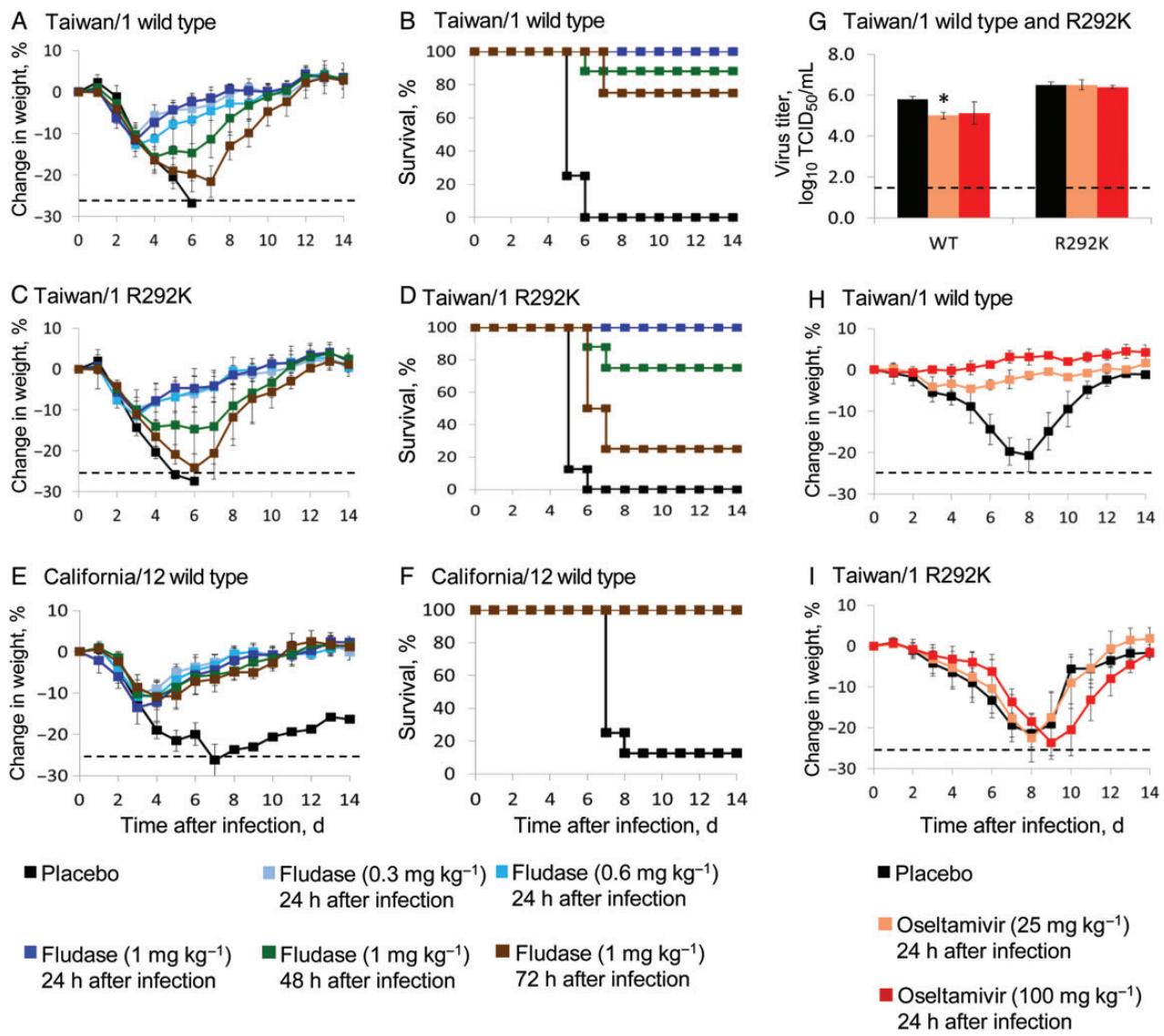
DAS181 is currently undergoing clinical evaluation as an inhalation drug to treat seasonal influenza. Here, the antiviral potency of DAS181 in the control of infection caused by avian influenza A(H7N9) recovered from infected patients in 2013, was evaluated in vitro and in a mouse model. We used 2 wild-type influenza A(H7N9) isolates (Shanghai/1 and Taiwan/1) and their counterparts carrying the NA R292K substitution. Our data show that the influenza A(H7N9) isolates replicated very efficiently in mouse lungs, regardless of the R292K presence. This result is in agreement with the report on a different influenza A(H7N9) pair, the wild-type A/Anhui/1/2013 (Anhui/1) and reassortant Anhui/1 virus carrying NA with R292K from Shanghai/1 virus [14]. Moreover, wild-type (R292) viruses and R292K virus variants maintained their NA sequence at residue 292, based on the data from a pyrosequencing assay for lungs collected on days 3 and 6 after infection. Of note, oseltamivir treatment did not result in acquisition of R292K by wild-type virus at both analyzed days.

As anticipated, the presence of R292K was accompanied by an increase in IC<sub>50</sub> values to Food and Drug Administration–approved (oseltamivir and zanamivir) and investigational (peramivir and laninamivir) NAIs in the NA inhibition assay,

consistent with the previous reports [8, 13, 14]. Although there are no established criteria for clinically relevant resistance of influenza viruses to the NAI class of drugs, the laboratory NA inhibition assay data for viruses carrying R292K may be interpreted as resistance to oseltamivir and, possibly, to 1 or more other NAIs, making it imperative to assess antiviral drugs with alternative mechanisms of action.

We found that replication of the R292K virus was not affected by treatment with oseltamivir in mice. Of note, the anti-influenza virus effect of late oseltamivir treatment was observed in mice challenged with lower inoculation dose of wild-type influenza A(H7N9). Conversely, the drug efficacy was compromised when a higher inoculation dose was used, despite early administration. Similarly, Watanabe et al [14] detected reduction in lung virus titers of wild-type Anhui/1 in mice (inoculation dose,  $10^3$  and  $10^4$  plaque-forming units) after high-dose oseltamivir treatment (started at 2 hours after infection) and little or no effect on the replication of R292K virus variant (reassortant Anhui/1). Furthermore, Baranovich et al [15] showed lung virus titer reduction in wild-type Anhui/1–infected mice (inoculation dose, 3 MLD<sub>50</sub> or  $10^{2.5}$  plaque-forming units) treated with high-dose oseltamivir, initiated 24 hours after infection. The published report and our present study highlighted the potency of oseltamivir treatment in mice infected with wild-type influenza A(H7N9).

Remarkably, treatment with DAS181 dramatically reduced the number of foci in cultured cells and lung virus titers in mice infected with the lethal dose of influenza A(H7N9), regardless of the presence of R292K. Although mice in the placebo-treated groups died from infection, once-daily treatment with DAS181 initiated at 4 hours after infection completely protected virus-infected animals from death, even at the lowest dose. Moreover, the DAS181 treatment prevented them from exhibiting severe disease signs, including body weight loss. Representative sera collected from DAS181-treated mice on day 21 after infection indicated that animals became infected and seroconverted (HI titer, 10–80) against influenza A(H7N9). Importantly, our data demonstrate that delayed treatment with DAS181, initiated 24–72 hours after wild-type virus infection, resulted in 75%–100% protection in mice. Lower survival rates were observed in R292K virus–infected animals given DAS181 48–72 hours after infection. On the other hand, all delayed DAS181 treatments prevailed when tested in mice infected with influenza A(H1N1)pdm09, consistent with the previous studies using influenza A(H1N1)pdm09 [12] or highly pathogenic avian influenza A(H5N1) [11]. Considering the high morbidity and substantial mortality in influenza A(H7N9)–infected mice that received DAS181 treatment at 72 hours, delay beyond 72 hours seems unlikely to produce significant beneficial effects in this model. As seen with the oseltamivir treatment, reducing the inoculation dose might improve clinical symptoms and survival rates of DAS181-treated animals. Taken together, once-daily treatment with DAS181 substantially



**Figure 2.** Efficacy of delayed treatment in mice. Animals (8/group) were intranasally inoculated with 5 50% mouse lethal doses of plaque-purified influenza A virus subtype H7N9, equivalent to  $1.6 \times 10^5$  50% tissue culture infective doses (TCID<sub>50</sub>) of Taiwan/1 wild-type (A, B) or  $1.6 \times 10^5$  TCID<sub>50</sub> of Taiwan/1 R292K (C, D). California/12 (a 2009 pandemic influenza A virus subtype H1N1 isolate;  $5 \times 10^4$  TCID<sub>50</sub>) was used as a control (E, F). Mice were treated with DAS181 24, 48, or 72 hours after infection (for a total of 6 regimens). In oseltamivir control groups, mice (n = 8) were infected with  $10^4$  TCID<sub>50</sub> of Taiwan/1 wild-type (G, H) or R292K (G, I) virus and received twice-daily treatment for 5 days, starting 24 hours after infection. Three mice were euthanized on day 6 after infection, and virus titers in lungs were determined by the TCID<sub>50</sub> assay in MDCK cells (G); dotted lines indicate the lower limit of virus titer detection ( $1.6 \log_{10}$  TCID<sub>50</sub>/mL). Body weights were monitored daily (A, C, E, H, I); animals that lost  $\geq 25\%$  (shown in dotted lines) of their initial body weights were humanely euthanized, and numbers of animals that survived are shown (B, D, F). P values of  $<0.05$  (asterisks) denote statistically significant differences from values for placebo-treated groups. Error bars indicate SDs.

reduced morbidity and avouched a high level of protection in mice, especially when given within 48 hours after lethal challenge with influenza A(H7N9).

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary

data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

**Acknowledgments.** We thank the Chinese Center for Disease Control and Prevention and Taiwan Centers for Disease Control, for sharing the A/Shanghai/1/2013 and A/Taiwan/1/2013 influenza A(H7N9) isolates, respectively; Ha Nguyen (Battelle), for participating in animal experiments; Daisuke Tamura (ORISE), Joyce Jones, and Todd Davis (CDC), for conducting sequence analysis; and Lester Slough (CDC), for excellent assistance with animal care.

**Disclaimer.** The findings and conclusions of this report are those of the authors and do not necessarily represent the views of the funding agencies or the Centers for Disease Control and Prevention.

**Financial support.** This work was supported by the Influenza Division, Centers for Disease Control and Prevention; and by an interagency agreement between Biomedical Advanced Research and Development Authority and the Centers for Disease Control and Prevention.

**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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